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Published in:
Journal of Mass Spectrometry

DOI:
10.1002/jms.288

Publication date:
2002

JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2002; **37**: 322–330
Published online 29 January 2002 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.288

JMS

Improved in-gel approaches to generate peptide maps of integral membrane proteins with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Received 7 September 2001; Revised 3 December 2001

This paper reports studies of in-gel digestion procedures to generate MALDI-MS peptide maps of integral membrane proteins. The methods were developed for the membrane domain of the mannitol permease of *E. coli*. In-gel digestion of this domain with trypsin, followed by extraction of the peptides from the gel, yields only 44% sequence coverage. Since lysines and arginines are seldomly found in the membrane-spanning regions, complete tryptic cleavage will generate large hydrophobic fragments, many of which are poorly soluble and most likely contribute to the low sequence coverage. Addition of the detergent octyl-β-glucopyranoside (OBG), at 0.1% concentration, to the extraction solvent increases the total number of peptides detected to at least 85% of the total protein sequence. OBG facilitates the recovery of hydrophobic peptides when they are SpeedVac dried during the extraction procedure. Many of the newly recovered peptides are partial cleavage products. This seems to be advantageous since it generates hydrophobic fragments with a hydrophilic solubilizing part. In-gel CNBr cleavage resulted in 5–10-fold more intense spectra, 83% sequence coverage, fully cleaved fragments and no effect of OBG. In contrast to tryptic cleavage sites, the CNBr cleavage sites are found in transmembrane segments; cleavage at these sites generates smaller hydrophobic fragments, which are more soluble and do not need OBG. With the results of both cleavages, a complete sequence coverage of the membrane domain of the mannitol permease of *E. coli* is obtained without the necessity of using HPLC separation. The protocols were applied to two other integral membrane proteins, which confirmed the general applicability of CNBr cleavage and the observed effects of OBG in peptide recovery after tryptic digestion.

KEYWORDS: membrane protein; peptide mapping; octyl glucoside; matrix-assisted laser desorption/ionization; sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abbreviations: MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; OBG, octyl-β-glucopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamidegel electrophoresis; CNBr, cyanogen bromide; HPLC, high-performance liquid chromatography; C domain, the membrane domain of the mannitol transporter without the soluble A and B domain; MscL, mechanosensitive channel of large conductance of *Escherichia coli*; LacS, lactose transporter of *Streptococcus thermophilus*.

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Contract/grant sponsor: Ministry of Economic Affairs.
Contract/grant sponsor: Ministry of Education, Culture and Science.
Contract/grant sponsor: Ministry of Agriculture, Nature Management and Fishery.
Contract/grant sponsor: Association of Biotechnology Research Netherlands (ABON).

INTRODUCTION

Since its invention, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has rapidly developed into a powerful peptide mapping tool for the direct analysis of peptide fragment mixtures, epitope mapping, localization of covalent modification, and protein identification.[1–3] In-gel digestion of polyacrylamide gel electrophoresis (PAGE) separated proteins has proved to be an efficient method to generate these peptide fragment mixtures.[4–7] However, most of the work has been done on soluble proteins rather than integral membrane proteins. The hydrophobic nature of these proteins necessitates the use of detergents for extraction and purification, but the presence of most of them leads to lower signal-to-noise ratios in MALDI mass spectra. In addition, charged residues, often the target site for proteases, are rarely found in the

membrane-spanning regions. This results in cleavage at the borders of membrane-spanning regions that are very difficult to solubilize. In the past, only the use of harsh conditions, such as unfolding with urea and guanidine, allowed the generation of complete peptide maps by HPLC in special solvent mixtures and subsequent Edman sequencing of the purified peptides.[8–10] Such conditions, however, are not compatible with the direct analysis of the digests with MALDI-MS and necessitate purification before the mass analysis. For instance, a nearly complete tryptic map of rhodopsin could only be obtained after removal of urea by reversed-phase HPLC purification with 0.1% octyl-β-glucopyranoside (OBG) in the solvents.[11] Similarly, complete CNBr maps of rhodopsin and bacteriorhodopsin could only be obtained after HPLC purification of peptides dissolved in pure TFA. In this case a large amount of delipidated protein without detergent was needed.[12,13] Recently, a protocol to generate complete CNBr maps from 30 pmol of rhodopsin, using 1–5 pmol of digest per sample, has been published.[14] Thorough purification of the protein by TCA precipitation and ethanol washing was essential to obtain the high sequence coverage.

The use of in-gel digestion in combination with MALDI-TOFMS resulted in maps of several porins, which covered more than 50% of the sequence, but substantial partial cleavage was observed.[15,16] An in-gel tryptic digestion approach also resulted in a very good map of the endothelin receptor from bovine lungs. Although many peptides were not fully cleaved, the combination of reversed-phase HPLC, MALDI-TOFMS and electrospray MS allowed the identification of one palmitoylated and several phosphorylated residues. The presence of 0.1% OBG in the HPLC was again essential.[17] A reasonably complete map of in-gel trypsin digested bradykinin B_2 receptor, which showed mainly partially cleaved fragments, has also been published.[18]

The in-gel approach, using small amounts of protein, seems to yield good peptide maps with high sequence coverage. The optimum situation, however, would be complete cleavage with picomole quantities of membrane protein and 80–90% sequence coverage. In this paper the complete coverage by MALDI-TOFMS of the membrane domain of an integral membrane protein, the C domain of the *E. coli* mannitol permease, EII^{mtl}, is presented. No separation by HPLC was needed. We used the MALDI-MS-compatible detergent octyl-β-glucopyranoside (OBG) to assist the recovery of hydrophobic tryptic fragments. In addition, we show that in-gel CNBr cleavage yielded spectra improved with respect to both intensity and completeness. The procedures were applied to two different integral membrane proteins, which confirmed the general applicability, the effect of OBG, and the results with CNBr.

EXPERIMENTAL

Materials

Chemicals and solvents used in this study were of analytical grade. The C domain of the mannitol permease is derived from a His-tagged mannitol permease (E. Vos, personal communication), the generation of which will be published elsewhere. This C domain contains three additional residues (glycine–serine–histidine) at the N-terminus and serines at positions 110 and 320 instead of the two wild-type cysteines. The sequence terminates at R350 in the original sequence; the C-terminal cytoplasmic A and B domains are thus removed.[19] The generation and purification of His-tagged mechanosensitive channel of large conductance of *E. coli* (MscL) will be described elsewhere. The purification of His-tagged lactose transporter of *Streptococcus thermophilus* (LacS), in which the C320 was replaced with an alanine, was done as described.[20] These proteins did not contain cysteines. Trypsin for in-gel tryptic digestions was sequencing grade modified trypsin from Promega. Laemmli sample buffer (2 × concentrate) and octyl-β-glucopyranoside (OBG) were purchased from Sigma. CNBr was obtained from Fluka.

SDS-PAGE

If necessary, protein samples were TCA precipitated by adding 10% (w/v) TCA and incubating at 0 °C for several hours. The pellets were washed twice with ice-cold acetone and allowed to air dry. Approximately 50 pmol of protein were solubilized in Laemmli sample buffer by heating at 95 °C and loaded on to the gel. To prevent aggregation, LacS was heated for 10 min at 50 °C. The samples were separated on Laemmli 15% SDS-polyacrylamide gels[21] or Bio-rad precast 4–15% Tris–glycine gels. The proteins were visualized with Coomassie Brilliant Blue staining.

In-gel tryptic digestion or CNBr cleavage

After visualization, the bands containing the protein were excised from SDS-polyacrylamide gels and completely destained with 50 mM ammonium hydrogencarbonate in 40% ethanol. These gel pieces were treated following the protocol of Hellman *et al.* [15] with some modifications. The excised gel pieces were washed with 200 µl of 25 mM ammonium hydrogencarbonate three times for 15 min and cut into pieces of <1 mm^3. Subsequently, the gel pieces were dehydrated with 100 µl of acetonitrile three times for 10 min and completely dried with a SpeedVac.

Tryptic digestion was started with the addition of 5 µl of 75 ng µl^{-1} trypsin in 25 mM ammonium hydrogencarbonate buffer to the dried gel pieces. After reswelling, these were covered with an overlay of ~20 µl of 25 mM ammonium hydrogencarbonate so that the gel pieces remained immersed throughout the digestion. In some experiments, to set up an optimized procedure, 2–4 M urea, 0.5 or 2% OBG or 40% acetonitrile was added to this overlay, but this did not improve the spectra, as stated in the Results section. These additions should not be added when the optimized procedures are being used. The protein was digested for at least 14 h at 30 °C without agitation. MALDI mass spectra were recorded directly from the overlay or after extraction (see later).

The CNBr cleavages were performed for at least 14 h in the dark at room temperature by adding 25 µl of CNBr in 70% TFA (one small crystal was dissolved in 200–300 µl of 70% TFA) to the dried gel pieces. Spectra were only recorded after extraction (see the next section).

Extraction of the peptides from the gel pieces

The digested gel pieces in the overlay were sonicated for 5 min in the absence or presence of 0.1% OBG. After collection of the overlay, the peptides were extracted twice by sonication for 5 min in 30 µl of 60% acetonitrile, 1% TFA in the absence or presence of 0.1% OBG. The overlay and extracts were pooled and dried in a SpeedVac. The last traces of ammonium hydrogencarbonate were removed by adding 10 µl of 1% TFA and subsequent drying in a SpeedVac.

Mass spectrometry

The dried samples were dissolved in 5 µl of 50% acetonitrile, 0.1% TFA and sonicated for 5 min. Aliquots of 0.5 µl were applied to the target and allowed to air dry. Subsequently, 0.5 µl of 10 mg ml^{-1} α-cyano-4-hydroxysuccinnamic acid in 50% acetonitrile, 0.1% (v/v) TFA was applied to the dried sample and again allowed to dry. MALDI mass spectra were recorded with a Bruker Biflex III MALDI time-of-flight mass spectrometer operated in the reflectron mode. The accuracy with external calibration was, in general, better than 200 ppm.

RESULTS

Tryptic digestion of the C domain of the mannitol permease

The integral membrane domain of the mannitol permease is very resistant to proteases, as evidenced by the fact that incubations in solution with normal up to relatively high concentrations of trypsin (1:1 ratio by weight) did not result in cleavage of this domain. Therefore, an in-gel approach was chosen which, in general, involves the following steps. A gel piece containing the protein of interest is excised, fully destained, cut into small pieces and dried after dehydration with acetonitrile. The tryptic digestion is started with the addition of a small aliquot of trypsin to the dried gel pieces, which results in reswelling of the gel. The gel pieces are then fully immersed with an overlay of ammonium hydrogencarbonate buffer and spectra of peptides diffusing out of the gel are recorded directly from the overlay. If necessary, peptides can be extracted from the gel pieces by sonication in organic solvent mixtures. The supernatant containing these extracted peptides can be pooled with the overlay, if desired, and is analyzed after concentration in a SpeedVac.

The predicted topology of the C domain[22] and its trypsin cleavage sites are shown in Fig. 1. Figure 2(A) shows a spectrum of the overlay of an in-gel trypsin-digested C domain. Table 1 shows the peaks in Fig. 2(A) that can be assigned to the C domain; these add up to 44% sequence coverage. Most of the other peaks in the spectrum could be attributed to trypsin-digested keratins. A peak at *m/z* 3945.9 could be assigned either to T4 or T10–11, which differ by only 0.1 Da in mass, but the resolution and peak intensity in this area of the spectrum did not allow unambiguous assignment. Extraction of the gel pieces without OBG by sonication in buffer followed by sonication in 60% acetonitrile, 1% TFA resulted in similar sequence coverage. No new peptides were observed, but the ratio between the lower and higher masses changed in favor of the higher masses, which suggests that the larger fragments diffuse less efficiently into the overlay (data not shown).

Table 1. MALDI-MS analysis of in-gel tryptic digested C domain: peaks are listed which were observed in spectra recorded directly from the overlay and after extraction in the presence of OBG

Peptide[a]	Residues	Expected mass[b] (MH^+)	Measured mass: overlay	Measured mass: extracted+ OBG
T2–3	10–17	934.55	934.45	
T2–4	10–53	4865.9[c]	4864.2[c]	4864.1[c]
T3	12–17	693.37	693.55	
T4[d]	18–53	3947.10	3945.91	3949.0[c]
T5	54–73	2119.22	2119.17	2119.21
T5–6	54–79	2730.56		2730.52
T5–7	54–115	6206.6[c]		6205.2
T6–7	74–115	4105.0[c]		4104.5[c]
T7	80–115	3490.80		3490.93
T7–8	80–119	4048.9[c]		4047.5[c]
T10–11[d]	125–162	3947.22	3945.91	3949.0[c]
T12	163–188	2800.46	2800.45	2800.47
T13	189–214	2860.55	2860.46	2860.54
T13–14	189–239	5592.6[c]		5590.6[c]
T13–15	189–243	5936.0[c]		5934.1[c]
T14	215–239	2747.41		2747.44
T14–15	215–243	3090.59		3090.55
T16	244–271	3071.59	3071.35	3071.56
T16–17	244–312	7006.5[c]		7002.7[c]
T18	313–338	2570.41		2570.35
T18–21	313–353	4245.9[c]		4245.4[c]
T20–21	342–353	1375.67	1375.56	1375.63
T21	344–353	1148.51	1148.37	

[a] Complete tryptic digestion of the C domain yields 21 peptides. Peptides arising from partial cleavage are shown as the component peptides.

[b] The monoisotopic single protonated mass of the peptides is shown.

[c] The average single protonated mass is taken.

[d] Peptide cannot be assigned unambiguously.

Improving the solubility during in-gel tryptic digestion

We rationalized that the main reason for the low peak intensity and incomplete sequence coverage could be the solubility of the peptides upon digestion and that improving the solubility might enhance the recovery. Trypsin is known to be tolerant to 2–4 M urea, 40% acetonitrile and OBG. When present in the overlay, urea did not improve the spectra; in fact, the urea digests could not be analyzed without prior removal of urea with a ZipTipC18 (Millipore). The digestions with 40% acetonitrile in the overlay were somewhat better with respect to intensity and reproducibility, but did not increase the sequence coverage.

The presence of 0.5 or 2% OBG in the overlay and subsequent extractions without OBG yielded spectra that

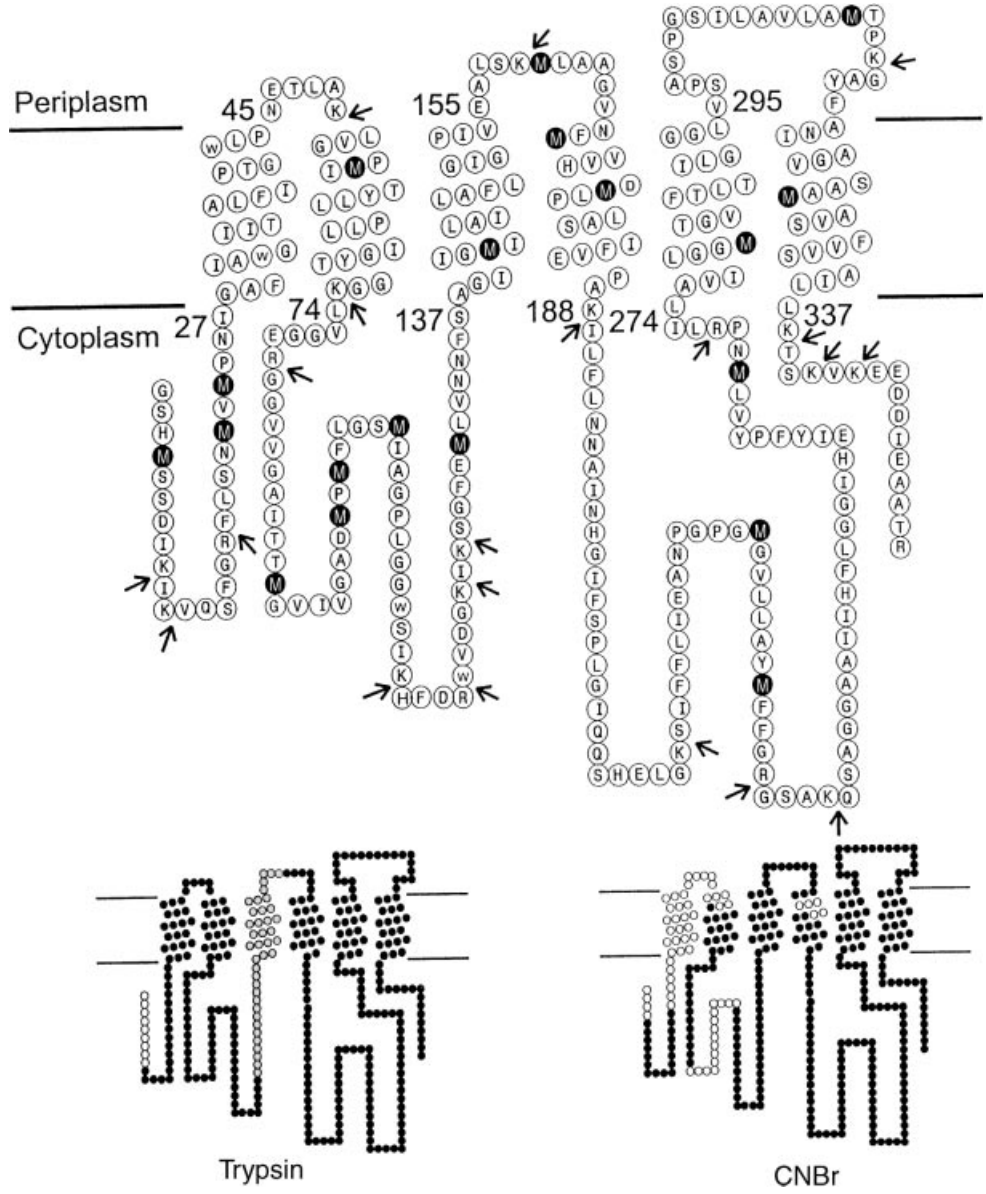

Figure 1. The cleavage sites of trypsin and CNBr indicated in the model of Sugiyama *et al*.[22] for the topological organization of the C domain of the mannitol permease. Trypsin cleaves at the C-terminal side of lysines and arginines as indicated by the arrows. CNBr cleaves at the C-terminal side of methionines, which are shown in black. The lower portion of the picture shows, on the left, the sequence coverage resulting from the tryptic digestion. Residues shaded gray were not unambiguously identified. The right shows the sequence coverage in the CNBr cleavages. Unfilled residues in both diagrams represent portions not identified as peaks in the mass spectra.

together added three peptides at *m/z* 2570.6 (T18), 2747.5 (T14) and 3490.9 (T7) to the sequence already covered, but the peptides with low *m/z* values were much less abundant (spectra not shown). These three peptides cover an additional 22% of the sequence. Spectra recorded directly from the overlays containing OBG did not show much signal.

The presence of OBG during the extractions

If OBG was added to the extraction solvents and not to the overlay, it was still possible to record spectra of the overlay without OBG as described above and, subsequently, spectra of peptides extracted with the help of OBG (see Fig. 2(B)). Additional peaks, also at *m/z* values above 3500, were observed, many of which could be assigned to the protein (see Table 1). Only 10% (5 pmol of protein) was used to obtain the spectra. Partial cleavage was also observed, providing overlapping fragments. The spectrum was not very intense below *m/z* 1500 but, in combination with spectra of the overlay, the sequence coverage increased to at least 85%. The lower portion of Fig. 1 shows the parts of the protein that were identified. The extraction with OBG thus increases the number of peaks observed. Figure 2(C) and (D) clearly show three peaks that appeared upon extraction with OBG. The peaks in this mass range that were already present in the extracts without OBG, such as T12 and T13, did not significantly increase in intensity. Figure 2(B) could also be obtained if OBG was not present in the extraction solvents but added to the mixture of extracted peptides before the

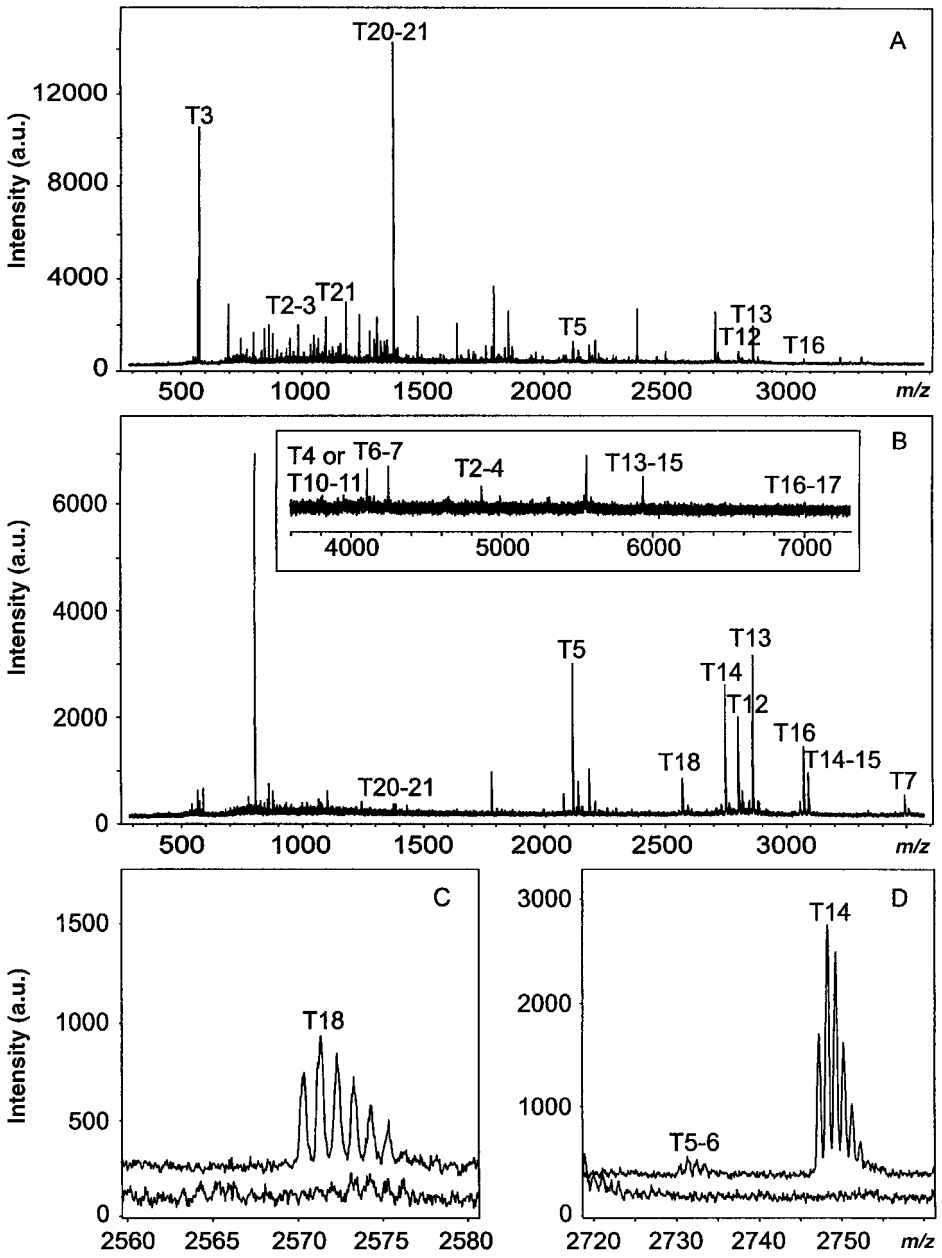

Figure 2. MALDI-TOF mass spectra of in-gel trypsin digested C domain of the mannitol permease. (A) Spectrum of a digest of the C domain, recorded directly from the overlay, which was used to immerse the gel pieces during digestion. (B) Spectrum of a digest of the C domain, recorded from an extract in the presence of OBG, which was pooled with the overlay before concentration in the SpeedVac. The inset shows the mass range *m/z* 3500–7500. Not all peptides that are present in the sample are indicated in the spectrum. Both panels (C) and (D) contain selected parts of two spectra of tryptic digests that were extracted either without or with OBG. The spectra show three peaks (T18, T5–6 and T14) that were clearly absent if OBG was not used.

SpeedVac drying. This shows that OBG was necessary in the SpeedVac drying and/or MALDI-MS analysis but not in the extraction itself.

CNBr cleavage of the C domain

The sequence coverage of the tryptic digests in combination with the assistance of OBG was already very good. Nevertheless, putative helix 3 was not unambiguously present in the spectra and might have been absent. Considering the amount of protein in the gel, the signal intensities were also rather low. Apparently a large part of the protein was not digested, not extracted from the gel pieces or not ionized in the MALDI. Therefore, the C domain was cleaved with CNBr. Figure 1 shows the location of the CNBr cleavage sites. The peptides were extracted in the presence or absence of OBG and mass spectra were recorded. Figure 3 shows a spectrum of a CNBr-cleaved C domain, extracted without OBG. The intensities of the signals were 5–10-fold higher than those of the tryptic digests. Many of the observed fragments could be assigned to the C domain and no partial cleavage was observed (Table 2). The sequence coverage was 83%. Hardly any modifications of the peptides were observed, except the oxidation of tryptophan in C9, which contains two tryptophans. The second most abundant peak near *m/z* 1500 could

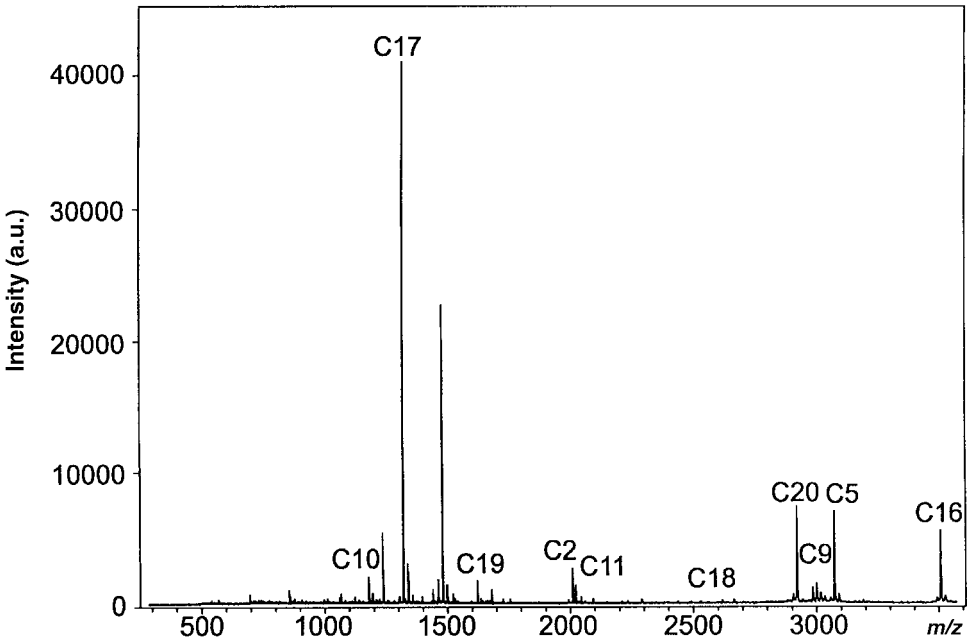

Figure 3. MALDI-TOF mass spectrum of in-gel CNBr-cleaved C domain of the mannitol permease. The peptides were extracted from the gel pieces without OBG. Not all peptides that could be assigned are indicated in the spectrum.

not be assigned, even when possible modifications such as oxidation, phosphorylation or acetylation were taken into account. At present we do not know what this peak represents. No difference in the spectra was observed if the extractions were done with or without OBG. Almost every peptide was present except C4 and some peptides with masses <800 Da. These small peptides were most likely suppressed by the matrix suppression settings of the mass spectrometer. The lower portion of Fig. 1 shows the protein sequence that was covered.

Peptide maps of MscL

To examine the general validity of the observations mentioned above, the mechanosensitive channel of large conductance of *E. coli*, MscL, was subjected to in-gel tryptic digestion and CNBr cleavage. MscL is a small integral membrane protein, which consists of two membrane-spanning α-helices and a cytoplasmic helix. The three-dimensional structure of a homologue is known.[23] Figure 4 shows the mass spectra of the extracts with OBG of a tryptic-digested and CNBr-cleaved sample. Partially cleaved tryptic fragments were observed and 66% of the protein sequence was covered. The second helix was not present in the spectrum of the tryptic digest [Fig. 4(A)]. This is understandable, since the corresponding tryptic fragment has an *m/z* of 3900, is very hydrophobic and hardly contains charged residues. The rest of the uncovered sequence consists of peptides smaller than 500 Da and, therefore, not present in the spectrum. The CNBr spectrum was much more intense, partially cleaved fragments were not observed and the complete protein sequence was covered.

Peptide maps of the lactose transporter

The lactose transporter of *Streptococcus thermophilus* contains a 50 kDa integral membrane domain with 12 putative membrane-spanning α-helices and a 21 kDa soluble cytoplasmic domain.[24] Figure 5 shows the mass spectra, which were generated with the procedures mentioned above. All the spectra of the tryptic digests, the overlay and the extract with and without OBG were dominated by peptides from the cytoplasmic domain. Peptides from the membrane domain could also be identified (T11 and T7), but these were much lower in intensity. The effect of OBG is again clearly visible in Fig. 5(B) and (C). T22 and T30 in Fig. 5(B) and (C) are peptides, which contain a membrane-spanning α-helix. These membrane-spanning peptides were not visible without OBG in the extract, but they were clearly visible when OBG was used. The peaks that were already present in the extracts without OBG did not increase significantly in

Table 2. MALDI-MS analysis of in-gel CNBr-cleaved C domain

Peptide[a]	Residues	Expected mass[b] (MH^+)	Measured mass
C2	5–22	2009.08	2009.05
C5	59–89	3071.75	3071.72
C9	105–131	2983.56	2983.31
C10	132–143	1187.64	1187.49
C11	144–163	2021.24	2021.24
C12	164–171	774.42	774.58
C14	177–228	5555.5[c]	5554.9
C15	229–235	718.41	718.64
C16	236–268	3506.84	3506.60
C17	269–281	1318.82	1318.84
C18	282–309	2594.48	2594.26
C19	310–326	1620.84	1620.82
C20	327–353	2919.60	2919.49

[a] Complete CNBr cleavage of the C domain yields 20 peptides.
[b] The monoisotopic single protonated mass of the peptides is shown.
[c] The average single protonated mass is taken.

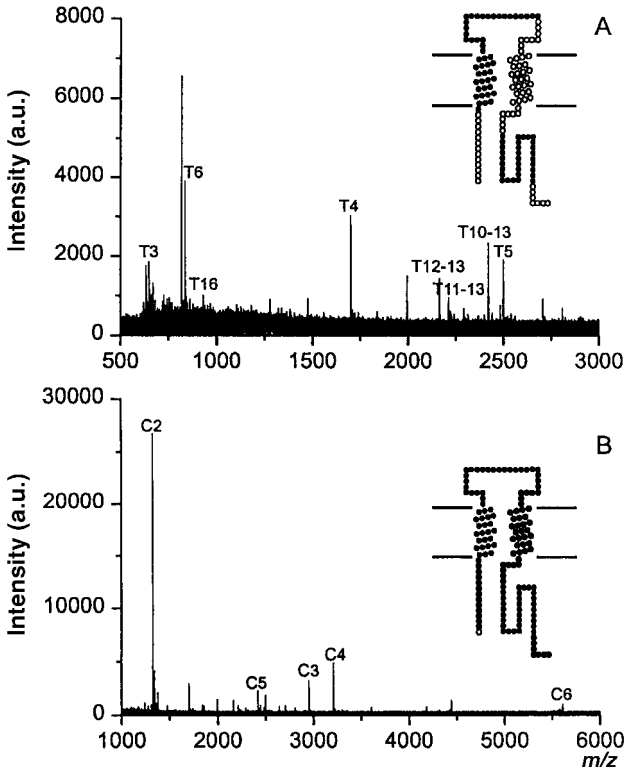

Figure 4. MALDI-TOF mass spectra of in-gel digested or cleaved MscL. (A) Spectrum of a tryptic digest of MscL, recorded from an extract in the presence of OBG, which was pooled with the overlay before concentration in the SpeedVac. Not all peptides that could be assigned are indicated in the spectrum. (B) Spectrum of CNBr-cleaved MscL, which was extracted in the presence of OBG. The insets show the sequence coverages in the topology model of MscL.

intensity (additional peak in Fig. 5(C)). The soluble domain contains only one methionine and CNBr cleavage would yield two very large fragments of ~7.5 and ~15 kDa, which are probably too large to be extracted from the gel and/or visible in the mass spectrum. The CNBr cleavage, therefore, only showed peptides from the membrane domain, which are of equal peak intensity as tryptic fragments of the soluble domain, but much more intense than tryptic fragments of the membrane domain. No significant effect of OBG was observed for the CNBr cleavages.

DISCUSSION

An in-gel approach to generate peptide maps of membrane proteins has several advantages. First, the amount of material that is needed is very small, convenient for the picomole quantities of membrane proteins readily obtained nowadays. Second, the purity of the sample is not critically important, because SDS-PAGE 'purifies' the protein. Third, many membrane proteins are resistant to proteolytic attack and first need to be unfolded with compounds that are not compatible with mass spectrometry. The in-gel approach combines the unfolding with boiling SDS-containing buffer and the simple removal of the denaturant. Finally, it is applicable for the identification of membrane proteins in proteomics studies.

The published in-gel digestion procedures used for membrane proteins thus far have been identical with the procedures used for soluble proteins. Considering the special properties of membrane proteins, it is not very likely that good results will be obtained for every membrane protein unless procedures are adapted to deal with them. The integral membrane domain of the mannitol permease is such an example. With the standard procedure, a relatively large amount of protein is needed to obtain a tryptic map, which covers less than 50% of the sequence. The observed intensities, however, are relatively low for such an amount of protein. Although this sequence coverage is more than enough for protein identification, it is not sufficient to elucidate modifications. Furthermore, these quantities of protein are higher than normally present in proteomics studies. Therefore, systematic studies to improve the procedures were necessary.

In-gel tryptic cleavage and the effect of OBG

The attempts to enhance the solubility with acetonitrile or urea during the tryptic digestion of the C domain did not really improve the peptide maps. High concentrations of OBG in the overlay did, however, result in the appearance of some new peaks. This was especially true with 2% OBG in the overlay but this also resulted in the suppression of the lower masses. This was also the case for the spectra obtained when extractions were carried out in the presence of 0.1% OBG, because SpeedVac drying and resolubilization resulted in an OBG concentration approaching 2%, well above the critical micellar concentration of 0.7%.[25] The masses that were already present in the extracts without OBG of the C domain and LacS were not significantly enhanced in the presence of OBG, suggesting that the effect of OBG is not a general effect on all of the peaks at higher *m/z* values. The suppression of the lower masses by very high concentrations of OBG in our experiments is not problematic, because it is possible to record mass spectra of the overlay before the extractions, in order to observe these smaller fragments. Alternatively, other matrix preparation procedures such as described by Cadene and Chait, for instance, might be capable of decreasing this suppression.[26] OBG was not necessary in the extraction itself, but it was essential during the SpeedVac drying and/or MALDI-MS analysis. It enhances the recovery of peptides after the SpeedVac drying. The effect is probably related to peptide solubility during the resolubilization and crystallization process, as has been suggested by others.[27] The results are confirmed by the data for MscL and LacS. It is clear, furthermore, that the analysis of LacS was severely effected by the presence of the soluble domain. This has been observed with an osmoregulated ABC transporter of *Lactococcus lactis*, which also contains a large soluble domain and an integral membrane domain (unpublished results). It is important to note that many membrane proteins contain such soluble domains. To obtain a high sequence coverage of the membrane domain, it might be worthwhile to remove the soluble domain before in-gel tryptic digestion. In general, these soluble domains are degraded by trypsin, whereas the membrane domains, in their native conformation, are not. Hence it is possible, in many cases, to remove the soluble

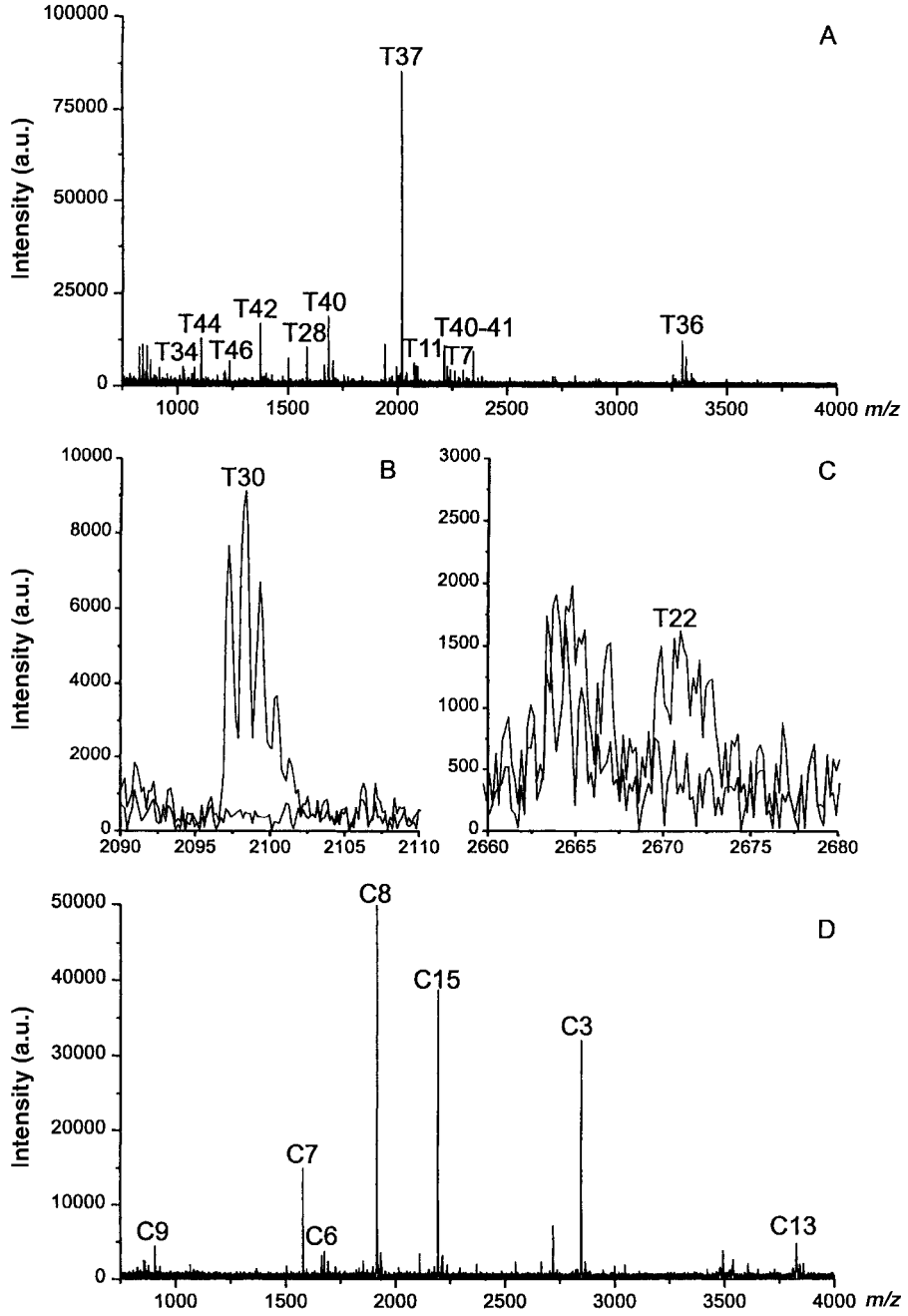

Figure 5. MALDI-TOF mass spectra of in-gel digested or cleaved LacS. (A) Spectrum of a tryptic digest, recorded directly from the overlay, which was used to immerse the gel pieces during digestion. (B) and (C) Both panels contain selected parts of two spectra of tryptic digests that were extracted either without or with OBG. The spectra show two peaks (T22 and T30) that were clearly absent if OBG was not used. (D) Spectrum of CNBr-cleaved LacS, recorded from an extract in the absence of OBG.

domains by proteolysis of the protein while still in the membrane or after purification but while it is still in its native conformation in detergent. A considerably simplified spectrum then results from the in-gel digestion of the remaining membrane domain under denaturing conditions.

Comparison between trypsin digestion and CNBr cleavage

Tryptic digestion and CNBr cleavage of the C domain of the mannitol permease both yielded spectra that covered a large portion of the sequence. However, many partially cleaved fragments were observed in the tryptic digestions, analogous to previously published tryptic peptide maps of other membrane proteins.[16–18] Since these partially cleaved peptides are composed of a large hydrophobic part and a hydrophilic part that probably acts as a solublizing tail, partial digestion might be advantageous by enhancing the solubility of the hydrophobic fragments. Some fully cleaved fragments were probably not very soluble and were lost; this could explain why the peak intensities were very low even though large amounts of protein were used. In contrast to trypsin cleavage sites, which are seldomly found in the transmembrane segments, the CNBr cleavage sites are also present in the membrane-spanning regions. Consequently, a transmembrane segment can be cleaved into smaller hydrophobic parts, which, in addition, can contain a

solubilizing hydrophilic part. OBG is probably not necessary for these fragments; indeed no effect of OBG on their recovery was observed.

Another reason for the low signal intensities of the trypsin digests might be the accessibility of the gel-trapped protein for trypsin. It is likely that the gel-trapped membrane proteins aggregate upon removal of the SDS and are, therefore, less accessible for trypsin. The limited accessibility is supported by the fact that peaks representing the small hydrophilic peptides of the C domain were also not very intense. The 70% TFA in the CNBr cleavages might more efficiently solubilize the proteins; this, plus the better accessibility of the gel-trapped proteins for CNBr, probably improves the cleavage efficiency.

Krause *et al*.[28] have suggested that tryptic peptides with a C-terminal arginine account for the most intense peaks in a MALDI spectrum. While this holds true in the spectrum in Fig. 2(A) for the fragments T3 and T20–21, it does not hold true for T16. Without actually determining the amount of peptide in each sample it is difficult to determine whether the low peak intensity of T16 is due to a smaller amount of material or to some other property specific to this peptide.

CONCLUSIONS

The results in this paper add new tools for the characterization and identification of membrane proteins by MALDI-TOFMS peptide mapping. The addition of OBG increases the sequence coverage of membrane proteins considerably. In-gel CNBr cleavage increases the intensity of the peaks and decreases partial cleavage. This is probably due not only to the higher solubilizing conditions during the cleavage, but also to the location of methionines in the membrane-spanning parts. The CNBr and tryptic maps together cover the whole sequence of the C domain of the mannitol permease and MscL without the necessity of using HPLC purification. The mass spectra of LacS do confirm the effects of OBG and CNBr cleavage. The methods presented here are generally applicable and will be useful in proteomics studies of membrane constituents.

Acknowledgements

We thank Guillermo Lopez Lluch for his help in the generation of the figures. Liesbeth Veenhoff, Mark Doeven and Robert Friesen are gratefully thanked for their assistance with the preparation of samples from LacS and MscL. We thank Bert Poolman for useful suggestions. This work was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science and the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research program of the Netherlands Association of Biotechnology Centers in the Netherlands (ABON).

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